SUSCEPTIBILITY AND RESISTANCE TO TRYPANOSOME INFECTIONS

I

ATTEMPTS AT IMMUNIZATION WITH DEAD AND ATTENUATED TRYPANOSOMES

BY

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Most observers who have had occasion to study malaria have been impressed by the fact that a recovery from an infection confers a certain degree of resistance. We have noted repeatedly that in communities in which malaria had been brought under control, but not completely eliminated, in other words, where the possibilities of re-infection are small, new infections develop chiefly among newcomers and relatively infrequently among the cured residents. Celli (1900), and Yorke and Macfie (1924) report instances of natural resistance to experimental malaria infections. There is little room for doubt, therefore, that there exists a type of immunity or resistance to malarial parasites, but the mechanism of this immunity or resistance is still obscure.

An experimental approach to this problem is difficult, because of the specific affinity of the malaria plasmodium for the human host. Consequently we have attempted to study the mechanism of susceptibility and resistance to a protozoon infection, the trypanosome, pathogenic for laboratory animals.

The *Trypanosoma evansi* which we have isolated from infected mules (1924) produces in rabbits a chronic relapsing infection. These animals are all susceptible to the infection and, apart from variations in individual animals, the nature of the disease which develops is fairly constant. There is an initial period of incubation followed by an intermittent fever, accompanied by slow emaciation and culminating in death. Various degrees of host resistance manifest themselves in longer or shorter incubation periods, in a more or less acute course of the disease and in a greater or lesser duration of the illness. Both the parasites and host are well adapted to the study we had in mind.

The Taliaferros (1922) have investigated the question of host resistance by following the course of events in the life-cycle of the invading parasite. They concluded from measurements of the parasites that in the relapsing type of infection the host in some way destroys the parasites, but does not affect the rate of reproduction.

In our study of this problem we have directed our observations particularly to the changes produced in the animal host. In a previous communication (1924) we showed : (1) That by the usual procedure it is not possible to demonstrate humoral parasiticidal antibodies ; (2) that there is a definite change in the leucocyte picture during the course of the disease, (3) that by disturbing the leucocyte balance by the injection of olive oil, a relapse or blood invasion may be produced at will ; and (4) that animals cured with ' Bayer 205 ' acquire an absolute resistance, of greater or lesser duration, to a re-infection.

On the basis of our observations we were led to conclude that the mechanism of infection and resistance, in the rabbit at least, is a resultant of the interaction of two elements, one generated by the host, the other by the parasite, and that the formed elements are chiefly responsible for host resistance. The conclusion reached was summarised by the following statement : ' In the course of the infection the resistance offered by the invasion of the blood stream is repeatedly broken down (probably by substances liberated by the parasites which continue their activity in the tissues), and trypanosomes penetrate into the circulation and go through a period of active development. The destruction of the trypanosomes in the circula-

tion leads to a partial immunization resulting in a disappearance of the parasites from the circulation.'

This assumption pre-supposes a double property of the parasitic cell; one sensitizing, the other immunizing. In this paper we present experimental evidence bearing on this point. The object of these experiments was to ascertain whether by the injection of dead and attenuated parasites it is possible: (1) To simulate the changes observed in the host during the natural course of an infection; and (2) to increase the resistance of the host to infection.

Experimental.

(A) Sensitization of animals by the injection of dead trypanosomes and trypanosome extracts.

Before presenting the experimental data a word of explanation is required as to the criteria which we have adopted for measuring increased resistance or susceptibility. The disease produced in experimental animals is, as already stated, invariably fatal. Increased susceptibility cannot, therefore, be measured by the percentage of animals dying and recovering from the infection, although that criterion may serve as a measure of increased resistance. In our preliminary experiments we found that the incubation period, that is, the interval between infection and the appearance of the trypanosomes in the circulation, is a dependable measure of the relative degree of that resistance. In other words, we used the resistance offered by the animal to blood invasion as an index of host resistance.

The incubation period as defined above is fairly constant for the rabbit, the animal used in these experiments. Sometimes a careful search may reveal a rare parasite in the peripheral blood within twenty-four hours after the infection. These disappear, however, the next day, and are not found again until the disease has established itself, as shown by the leucopenia and the increase of the mononuclears to about 60 per cent. of the total.

In the blood examinations we always used the thick drop, stained with Giemsa solution (1 : 20) for thirty minutes. At least three drops are examined and the results are recorded as the average number of trypanosomes per microscopic field. This method enables us to gauge roughly the relative intensity of infection as well as the presence

or absence of trypanosomes. The mere presence of a rare trypanosome (less than one in twenty fields) is not considered as a positive blood invasion.

Experiment 1. Heavily infected guinea-pigs were bled into citrate solution, the red blood cells sedimented by centrifugalization at slow speed (600 revolutions per minute, for ten minutes) and the supernatant fluid removed and centrifuged at high speed (1,500 to 2,000) for fifteen minutes. The clear fluid was decanted and the sediment washed with saline, and again centrifuged. The clear supernatant serum and the saline washings were mixed and injected intravenously into rabbits. The sediment was suspended in 20 c.c. of sterile distilled water, frozen and thawed several times and then injected intravenously into rabbits. Each animal received three injections at five-day intervals, and two weeks after the last injection the animals were inoculated with infected guinea-pig blood. The details of the procedure and results are summarised in the protocols given below.

Rabbit	Weight	Material injected	Dates	Date of infection	Incubation	External symptoms	Character of infection
30	grams 1,910	Supernatant fluid	20th July 25th July 30th July	13th Aug.	4 days	Swelling of ears and eyelids 5 to 7 days after infection; marked after 17 days; swelling intermittent.	Moderate intermittent; after first attack blood invasion uncommon.
31	1,720	Supernatant fluid	20th July 25th July 30th July	13th Aug.	3 days	Swelling of ears and eyelids 5 to 7 days after infection; marked after 17 days; swelling intermittent	After first attack of 2 days, blood invasions absent 2 weeks
32	1,350	Autolized sediment	20th July 25th July 30th July	13th Aug.	4 days	Swelling of ears and eyelids; marked after 2 weeks	Severe infection first 5 days; followed by rare, mild recurrences
33	1,600	—	"	"	"	"	"
34	1,760	*Control:* Supernatant serum; normal g.-p. blood	20th July 25th July 30th July	13th Aug.	8 days	No swelling	Mild intermittent infection at first, later more severe
35	1,675	*Control:* Sediment; normal g.-p. blood	20th July 25th July 30th July	13th Aug.	10 days	No swelling	Mild intermittent infection at first, later more severe

This experiment was repeated with essentially the same results, irrespective of whether infected guinea-pig or rabbit blood was used, in infecting the treated adimals. The rabbits receiving the autolized sedimented trypanosomes or the washings had a shorter incubation

period, a severe blood invasion during the first few days, followed by rare recurrences; while the control animals developed the usual intermittent infection, mild at first and more severe as the disease progressed, after an incubation period two to three times as long as that in the treated animals. In the treated rabbits the swelling of the ears and eyelids, which was sometimes extremely severe, was a constant accompaniment.

Experiment 2a. A second series of experiments was undertaken to ascertain the effect of the repeated injection of whole trypanosomes in increasing the resistance or susceptibility of the host to infection. The material was obtained as before from the blood of heavily infected guinea-pigs. The blood was collected in citrate solution and allowed to sediment overnight. The trypanosomes in the supernatant fluid were sedimented, re-suspended in saline and killed by heating to 54° C. for thirty minutes. The animals were given five sets of daily injections, in five-day periods, with intervals of two days, or a total of 25 injections.

The results are shown in the protocols below. The effect of these repeated injections was even more severe than that produced by the three injections of autolized trypanosomes. The incubation period was greatly reduced, the blood infection during the first few days was severe and swelling appeared promptly with the onset of the illness and then subsided. In general the indication is that the injection of dead or autolized trypanosomes leads to an explosive onset which subsides after a few days, and is then followed by the usual or an even milder course of the disease.

Rabbit	Weight*	Injections	Infected	Incubation	Swelling	Character of infection
	grams					
50	1,525	25: from 6th Nov. to 9th Dec.	21st Dec. (0·2 c.c. g.-p. blood, 2 tryps. per field)	1 day	Pronounced first day, then subsided; intermittent; severe few days before death	Severe, continuous 7 days, then only 2 recurrences in a month. Died 40 days after infection
51	1,370	*Control*	21st Dec. (0·2 c.c. g.-p. blood, 2 tryps. per field)	5 days	Swelling of ears and eyelids moderate after 45 days	Moderate, intermittent. Died after 60 days
52	1,160	5th Dec. to 7th Jan.	21st Dec. (0·2 c.c. g.-p. blood, 2 tryps. per field)	1 day	Pronounced first day; eyelids, ears and mouth; subsided after 5 days	Severe 4 days; then no relapses until 2 days before death, on 3rd Feb.
53	1,075	Serum and washing from material used for g.-p. 52; 5th Dec. to 7th Jan.	—	2 days	Swelling of ears and eyelids on second day; subsided fifth day	Heavy 3 days; then moderate. Died 5th March
54	1,100	*Control*	—	6 days	None	Heavy, died 2nd Feb.

* In so far as possible the weights of the animals in any experiment were approximately the same, the variation being 100-150 grams.

Experiment 2b. This experiment was the same as the previous one, except that larger rabbits were used, and only 20 injections were given; in addition to the normal control there was also a control rabbit, which received injections of sediment from normal guinea-pig blood. The results were essentially the same as those of the previous experiments.

Rabbit	Weight*	Injections	Infected	Incubation	Swelling	Character of infection
	grams					
90	1,850	Sedimented; heat killed tryps.; 20 injections ended 10th April	22nd April	4 days	Pronounced after 5 days; intermittent	Continuous 6 days, then intermittent
91	2,000	Sediment normal; g.-p. plasma; 20 injections	22nd April	7 days	Slight after 20 days	Moderate intermittent
92	1,900	Untreated	22nd April	8 days	Slight after 16 days	Moderate intermittent

* In so far as possible the weights of the animals in any experiment were approximately the same, the variation being 100-150 grams.

Experiment 3. This series of experiments varied from the previous one in the preparation of the injecting material. In order to eliminate the possibility of autolysis which might occur while standing overnight, the citrated blood was promptly sedimented, the trypanosomes in the supernatant fluid thrown down at high speed, re-suspended in saline heated at 54° C. for thirty minutes and injected. The whole operation required about one hour. The number of injections was the same as in Experiment 2a, and the effect essentially the same.

Rabbit	Weight	Number of injections	Incubation	Swelling	Infection
	grams				
114	1,800	25	2 days	Moderate for 3 to 5 days after infection; then subsided	Moderate intermittent
115	1,700	25	2 days	Moderate for 3 to 5 days after infection; then subsided	Moderate intermittent
116	1,790	None	6 days	None	Moderate intermittent

Experiment 3b. The same experiment was repeated giving only 10 injections instead of 25. The difference between the control and treated animals was imperceptible.

It seems clear from the results of these experiments that the repeated injection of whole or autolized trypanosomes fails to increase the resistance of the animal, but on the contrary renders it hypersensitive in so far as invasion of the peripheral blood stream is concerned. When autolized trypanosomes (obtained by freezing

and thawing) are employed, three injections of moderately large doses suffice to produce a marked hypersensitiveness. The same results may also be obtained by a sufficient number of injections of the supernatant serum and washing of large numbers of whole trypanosomes. But the most striking effect is produced by a long series of injections of whole heat-killed trypanosomes. These injections seem in no way to have modified the later course of the disease, except that the treated animals more often showed marked swelling and oedema, a condition which is found in untreated animals only after the disease has progressed for some time and become chronic.

In order to get some clue to the concomitant changes occurring in the animal host during the course of the infection, we studied the blood changes during the preparatory treatment, pre-infection and post-infection stages, as well as the cellular reaction in the peritoneal cavity subsequent to the infection. The results fluctuated considerably and gave no indication of any significant changes in the white blood counts.

The immediate effect of the injection of trypanosomes, serum, or autolized trypanosomes, was an increase in the total leucocyte count and in the polynuclears. The total leucocyte count was sometimes double the normal. This first reaction was followed by a progressive decrease in the polynuclears and by a proportional increase in the lymphocytes, the total count remaining the same. Each subsequent injection produced the same effect—an immediate rise in the polynuclears followed by a fall. Usually, on about the fifth day after the injection there is a tendency to return to the normal ratio, which is reached in about eight days. Control animals injected with normal guinea-pig serum or sediment show the same general tendency but not to such a marked degree. This disturbance of the white cell ratio is evidently due to the injection of foreign material and is not a specific reaction to the trypanosome cell. The only constant change due to the trypanosome infection is the one previously noted, namely the lymphocytosis, which accompanies the development of the infection, and is most marked during periods of blood invasion.

The changes in the peritoneal fluid were also apparently non-specific. After the first twenty-four hours polynuclears predominate

(70 to 80 per cent.), then there is a progressive increase in the proportion of lymphocytes, so that on the fourth or fifth day they constitute about 90 per cent. of the total. There is no apparent relation between this reaction, the previous treatment of the animal and the activity of the injected trypanosomes in the peritoneal cavity. In some animals the trypanosomes continue multiplying in the peritoneum for several days; in others they disappear from the cavity in twenty-four hours.

(B) Resistance of animals treated with trypanosomes suspended in a solution of 'Bayer 205.'

Failing to induce any sort of immunity by repeated injections of trypanosomes, either intact or autolized, we turned our attention in another direction. Experiments already reported (1924) showed that infected animals cured with 'Bayer 205' developed an immunity to reinfection which was more constant and of longer duration than the resistance of healthy animals treated with the same dose of the drug. It appeared, therefore, that the resulting immunity was induced by a combined action of the trypanosomes and the drug. Consequently a series of experiments was carried out to determine whether a similar effect could be obtained by treating animals with a mixture of dead or attenuated trypanosomes and 'Bayer 205.'

Experiment 4. Blood from heavily infected guinea-pigs was withdrawn into citrate solution and sedimented, first at low speed to remove the red cells, then at high speed to collect the trypanosomes. The sedimented trypanosomes were re-suspended in saline, and frozen and thawed four times. The material was divided into two parts; one part was injected directly into a rabbit, the second portion was added to a solution of Germanin ('Bayer 205'), kept for half-an-hour at room temperature, and then injected into another rabbit. A control rabbit received an equivalent amount of the drug without trypanosomes, repeated on three consecutive days. The total quantity of drug injected was 0·005 gm. per kilo, an amount shown by our previous experiments (1924) to be insufficient to protect a rabbit against infection. Two weeks after the last injection the animals were infected with the same dose (0·1 c.c.) of guinea-pig blood.

The results were striking. The animal which received injections of trypanosomes alone had a severe explosive infection, similar to those recorded above, resulting in death after fifteen days. Trypanosomes were constantly present in the blood, beginning with the second day.

The rabbit which received only 'Bayer 205' had a prolonged incubation period (ten days), and a moderate infection ending in death on the sixteenth day.

The rabbit which received trypanosomes plus ' Bayer 205 ' seemed at first, from the nature of the differential leucocyte count, to be developing an infection (on 24th October the differential white cell count was polynuclears 40, mononuclears 60) ; but it recovered completely, as was indicated by the increased weight of the animal and the return of the normal differential count.

The resistance was of relatively short duration. On 28th November, after the rabbit had completely recovered, it was re-infected and after a somewhat prolonged incubation period of ten days it developed a typical infection, which ran the usual course.

The details of the experiment are tabulated on p. 156.

Experiment 2. The second experiment was similar to the previous one, except that the trypanosomes were not disintegrated by freezing and thawing, but were rendered non-infective by exposing them for twenty-four hours to the action of dilute solution of ' Bayer 205.' Heavily infected guinea-pigs were bled into citrate solution and the mixture centrifugalized at low speed (600 revolutions per minute) for ten minutes. To the clear plasma, containing large numbers of trypanosomes, ' Bayer 205 ' was added to make a final dilution of 1 : 400, and the tubes incubated at 25° C. for twenty-four hours. This treatment leaves the trypanosomes intact, but partially or wholly immobilizes them and renders them non-infective. A control rabbit received only ' Bayer 205 ' in the same dilution and amount. Four injections were given on alternate days.

This experiment was not as conclusive as the previous one, because by an error the total amount of ' Bayer ' was 0·01 gm. per kilo instead of 0·005 gm., and this quantity masked the effect of the trypanosomes. Both animals, the one treated with trypanosomes plus ' Bayer 205 ' and the one treated with the drug alone recovered from the infection, with the difference that no trypanosomes were ever found in the peripheral blood of the former, while in the latter they were present once, on the seventh day of the infection, and then disappeared.

Experiment 3. In this experiment 10 c.c. of heavily infected blood was drawn from guinea-pigs, the red cells sedimented, the plasma decanted and the trypanosomes thrown down at high speed. The sedimented trypanosomes were re-suspended in saline, heated to 55° C. for five minutes, 0·002 gms. ' Bayer 205 ' added, and the material injected intravenously into a rabbit. Three injections were given on three successive days. A control rabbit received injections of comparable doses (a total of 0·005 gms. per kilo) of ' Bayer 205 ' alone. Ten days later the animals were infected.

The results are seen in the following protocol. The control rabbit receiving no treatment developed the usual infection after

Rabbit	Weight	Material injected	Dates	Date of infection	Result	White blood counts; average for five-day period	Differential counts; average for five-day period	Results
	grams						15–19 20–24 24 +	
K	820	Sedimented tryps., frozen and thawed	25th Sept. to 27th Sept.	12th Oct.	14th Oct., blood positive; heavy infection; continuous until death, 27th Oct.	7,500; 6,500	*P*38, *M*62; *P*37, *M*62; *P*35, *M*65	Died 27th Oct.
L	895	Tryps. as above; + total 0·005 gm. 'Bayer 205' per kilo.	25th Sept. to 27th Sept.	12th Oct.	Infection negative; animal gained in weight	7,300; 7,400; 7,400	*P*48, *M*52; *P*44, *M*55; *P*43, *M*57; *P*46, *M*54; *P*52, *M*48	Discharged 20th Nov.; Weight, 1,085 gm.; W. B. C. 9,300; Differential, *P*55, *M*45
M	850	Only 'Bayer 205'; total 0·005 gm. per kilo. given in 3 injections of 0·0014 gm.	25th Sept. to 27th Sept.	12th Oct.	Blood positive, 22nd Oct., after 10 days	7,800; 7,800	*P*50, *M*50; *P*43, *M*57	Died 28th Oct.

P = Polynuclears. *M* = Mononuclears.

an incubation period of seven days, the disease following the regular course with a fatal ending on 20th June (88 days). In the rabbit treated with 'Bayer 205' alone trypanosomes appeared in the peripheral circulation six days after the inoculation and the disease followed the usual course. The animal died on 4th May, from an intercurrent infection.

The rabbit treated with trypanosomes plus 'Bayer' had a prolonged incubation period of fourteen days and a milder infection which ended on 15th July (113 days).

In this experiment there is again an absence of the hypersensitive condition noted in animals treated with trypanosomes alone, and a degree of resistance not observed in the control animal, treated with the corresponding dose of the drug without trypanosomes, despite the fact that the latter was larger and consequently possessed greater natural resistance.

Rabbit	Weight	Treatment, 11th March to 13th March	Infected	Incubation	Death	Nature of infection
	grams					
56	1,300	None	24th March	7 days	88 days	Moderate
57	1,900*	Three injections of 'Bayer 205' totalling 0·005 gm. per kilo.	24th March	6 days	Intercurrent infection, 56th day	Moderate
58	1,280	Three injections of trypanosomes heated at 55° C., plus 'Bayer 205,' totalling 0·005 gm. per kilo.	24th March	14 days	113 days	Mild

* An animal of the same weight was not available at the time of the experiment and consequently a larger rabbit was used as the drug control.

Experiment 4. This experiment followed the same general plan as the previous one, with variations in details. Two rabbits received ten daily injections of trypanosome suspensions in saline. The trypanosomes were obtained from infected guinea-pigs, sedimented, suspended in saline and killed or attenuated by incubating at 37° C. for twenty-four hours. Two more rabbits received the same number of injections of material prepared in the same way, except that 'Bayer 205' was added in amounts so graduated that the total quantity injected should not exceed 0·005 gm. per kilo. A fifth rabbit received ten daily injections of 'Bayer 205', totalling 0·005 gm. per kilo.

The results corresponded with those obtained in the previous experiments. The animals treated with trypanosomes alone developed the blood infection on the fourth and fifth day after the

inoculation, with mild swelling of the eyelids. Those receiving both trypanosomes and ' Bayer ' developed the blood infection only after nineteen and twenty-one days respectively and swelling appeared only towards the end of the disease. The animal treated with small doses of ' Bayer 205 ' alone developed the infection on the eleventh day.

Rabbit	Weight	Treatment	Duration of treatment	Infected	Onset of disease*	Death
	grams					
64	1,170	Ten injections killed trypanosomes	14th April to 25th April	6th May	10th May	29th May, intercurrent infection
65	1,000	Ten injections killed trypanosomes	14th April to 25th April	6th May	11th May	12th June, typical
66	1,250	Ten injections trypanosomes plus Bayer	14th April to 25th April	6th May	25th May	3rd July, typical
67	1,050	Ten injections trypanosomes plus Bayer	14th April to 25th April	6th May	27th May	24th June, intercurrent infection
68	1,100	Ten injections ' Bayer 205 '	14th April to 25th April	6th May	17th May	15th July, typical

* Trypanosomes in peripheral circulation.

DISCUSSION

On account of technical difficulties experienced in collecting large amounts of trypanosomes with the primitive apparatus available, these experiments had to be temporarily suspended. Enough work has been done, however, to indicate that trypanosomes suspended in ' Bayer 205 ' behave quite differently from those suspended in saline and that the animals so treated, instead of becoming hypersusceptible, develop a resistance quite distinct from that induced by the same quantity of the drug alone.

The experiments reported above give a clue to the nature of some of the pathological changes noted in trypanosome infection, at least in so far as they manifest themselves in experimental animals. The mechanism of the disease is evidently a complex one. First there is the unexplained mystery of the appearance and disappearance of parasites in the blood stream, then there are the late

manifestations of the disease, varying from mild swelling and oedema to severe ulceration and necrosis.

In so far as the experiments reported in this paper simulate the changes occurring in the infected animals, it appears that the manifestations of the disease are due to a progressive sensitization of the host by the parasite or its product. The periodic invasion of the blood stream seems to depend on the repeated breaking down of some barrier by the trypanosomes multiplying in the animal organism. Just as the repeated injection of whole, or disintegrated, trypanosomes sensitizes the animals so that when infected the invasion of the circulation is prompt and, in small rabbits, even continuous, so in infected normal animals a period of incubation is required for sensitizing the animal before the peripheral circulation can be invaded.

Similarly the appearance of swelling and oedema is due to a prior sensitization by the trypanosomes or their products. In healthy animals this condition can be produced artificially by the injection of the same strain of trypanosomes, killed, attenuated or disintegrated. In animals previously so treated, swelling of the ears and eyelids occurs promptly after the infection simultaneously with the invasion of the circulation. In normal animals swelling occurs only late in the disease, the fourth or fifth week ; or, in other words, after the animal has become spontaneously sensitized by the absorption of the parasites or their products. The oedema and swelling noted in the later stages of the disease are, therefore, direct effects of the parasite and its products.

If, however, the condition of treatment is varied and the trypanosomes are injected together with the drug ' Bayer 205,' an exactly opposite state is established in the animals. Instead of being hypersensitive, the animals manifest an increased resistance which in some of them may suffice to ward off an infection. This acquired resistance cannot be attributed to the protective action of the drug, because the same dose of the drug without trypanosomes has little or only a mild protective action.

The same phenomenon has already been observed (1924) in infected animals treated with ' Bayer 205.' Animals cured with this drug manifest a degree of resistance to re-infection greater and of much longer duration than normal controls treated with corresponding doses of the drug.

It is difficult to imagine what peculiar transformation is brought about by the drug which results in such markedly different reactions. It would appear that the drug combines in some way with the trypanosome cell, producing a heterogenic antigen capable of stimulating the formation of specific antibodies.

This may possibly be a demonstration of the type of a combined chemico-immuno-therapy supposed by Yorke (1925) to be going on in malaria patients treated with quinine. The latter drug is just as specific for the malaria plasmodia as ' Bayer 205 ' is for trypanosomes. The action of both these drugs is not, however, purely germicidal ; associated with this property there is apparently also an immunizing activity which completes the cure and affords an increased resistance of greater or lesser duration to subsequent infection. Whether or not artificial immunization would result from the treatment of suitable animals with Haemosporidia and quinine remains to be determined ; the experiments reported here, incomplete though they admittedly are, suggest that animals may be rendered more resistant to trypanosome infection if treated with a mixture of trypanosomes and ' Bayer 205.'

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